A comprehensive LC-QTOF-MS metabolic phenotyping strategy: application to alkaptonuria

Running Head: Comprehensive metabolic phenotyping by LC-QTOF-MS

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Abbreviations: LC – liquid chromatography; QTOF-MS – quadrupole time-of-flight mass spectrometry; AMRT - accurate mass/retention time; MS – mass spectrometry; AKU – alkaptonuria; HGD – homogentisate 1,2-dioxygenase; HGA – homogentisic acid; NAC – National Alkaptonuria Centre; RT – retention time; QC - quality control; CV – coefficient of variation; FC – fold change; HT-1 - hereditary tyrosinaemia type-1

List of human genes: *HGD* - homogentisate 1,2-dioxygenase

Abstract

Background: Identification of unknown chemical entities is a major challenge in metabolomics. To address this challenge, we developed a comprehensive targeted profiling strategy, combining three complementary liquid chromatography (LC) quadrupole time-of-flight mass spectrometry (QTOF-MS) techniques and in-house accurate mass retention time (AMRT) databases established from commercial standards. This strategy was used to evaluate the effect of nitisinone on the urinary metabolome of patients and mice with alkaptonuria (AKU). Because hypertyrosinemia is a known consequence of nitisinone therapy, we investigated the wider metabolic consequences beyond hypertyrosinemia.

Methods: 619 standards (molecular weight 45-1354 Da) covering a range of primary metabolic pathways were analyzed using three LC methods, two reversed phase and one normal phase, coupled to QTOF-MS. Separate AMRT databases were generated for the three methods, comprising chemical name, formula, theoretical accurate mass and measured retention time. Databases were used to identify chemical entities acquired from non-targeted analysis of AKU urine; match window theoretical accurate mass ±10 ppm and retention time ±0.3 min.

Results: Application of the AMRT databases to data acquired from analysis of urine from 25 patients with AKU (pre-treatment and after 3, 12 and 24 mo on nitisinone) and 18 *HGD*$^{-/-}$ mice (pre-treatment and after one week on nitisinone) revealed 31 previously unreported statistically significant changes in metabolite patterns and abundance, indicating alterations to tyrosine, tryptophan and purine metabolism post-nitisinone.

Conclusions: The comprehensive targeted profiling strategy described here has the potential of enabling discovery of novel pathways associated with pathogenesis and treatment of AKU.

Metabolic profiling has potential to advance knowledge of disease beyond established biochemical pathways and will play a major role in precision medicine (1-5). However, metabolite identification is a major challenge in untargeted profiling studies using mass spectrometry (MS) (6,7) since they generate many chemical signals representing 'unknowns'. To address this challenge, we generated three accurate mass/retention time (AMRT) databases from 619 metabolite standards using liquid chromatography-quadrupole time-of-flight-mass spectrometry (LC-QTOF-MS). We applied this strategy to the inborn error of metabolism alkaptonuria (AKU, OMIM 203500). In AKU, bi-allelic mutations in the homogentisate 1,2-dioxygenase (*HGD*) gene result in a lack of homogentisate 1,2-dioxygenase (HGD, E.C.1.12.11.5) (8), leading to increased homogentisic acid (HGA), a metabolite of tyrosine catabolism (Fig. 1). HGA accumulates in connective tissue, particularly cartilage, where it is deposited as a dark pigment, a process termed ochronosis. Ochronosis underlies a range of clinical features in AKU, the most debilitating of which is severe, early-onset osteoarthropathy that alters the physico-mechanical properties of cartilage (9,10).

Nitisinone has emerged as a promising therapeutic agent in AKU because it reduces circulating HGA concentrations (11–15) by reversibly inhibiting hydroxyphenylpyruvic acid dioxygenase (E.C. 1.13.11.27). Nitisinone completely inhibits ochronosis in an *HGD*$^{-/-}$ mouse model of AKU (16). Although not currently licensed for AKU, nitisinone is being used to treat patients attending the National Alkaptonuria Centre (NAC) in the UK. One of the major metabolic consequences of nitisinone is hypertyrosinaemia (11,13–15,17–21) (Fig. 1). The objective of our study was to apply the developed profiling strategy to urine from patients with AKU and HGD$^{-/-}$ mice to understand the wider metabolic consequences of nitisinone treatment.

Materials and Methods

Metabolite standards library preparation

619 standards (IROA Technology MS metabolite library of standards; molecular weight 45-1354 Da) were from Sigma-Aldrich (UK) in seven deepwell plates. Each compound was present at 5 µg/well (>95% purity). Plates were stored at -80°C. The standards covered a broad range of primary and intermediary metabolism, including the following compound classes: carboxylic acids, amino acids, biogenic amines, polyamines, nucleotides, coenzymes, vitamins, carbohydrates, fatty acids, lipids, steroids and hormones (Supplemental Files 1 and 2).

Prior to analysis, plates were thawed at ambient temperature (18˚C) and compounds reconstituted in 15 µL methanol (LC/MS grade, Sigma-Aldrich) followed by 285 µL deionized water (DIRECT-Q 3UV Millipore water purification system). Plates 1-5 were left to stand for 1 h following addition of water and methanol. Plates 6 and 7 (primarily non-polar compounds) were left to stand for 2 h at room temperature following addition of methanol to ensure solubilization; water was then added. Plates were agitated on a plate shaker (MTS 2/4m IKA, Germany) at 600 rpm for 10 min. Compounds were pooled across rows of each plate (12 wells/row). 20 µL was removed from each well and pooled into one well (12 compounds analyzed per injection). Fifty-six injections were performed in positive and negative polarity across all plates (112 injections for each LC-QTOF-MS method; Supplemental Fig. 1). The total volume of each pool was 240 µL with each compound at 14 mg/dL (1-31 µmol/L).

Evaluation of LC-QTOF-MS strategy and effect of nitisinone on the metabolome using AKU urine

24 h urine was obtained from 25 patients with AKU attending the NAC (12 male, mean(±SD) age 51±15 y). Samples were collected before nitisinone then at 3 (2 mg every other day), 12 and 24 (2 mg daily) mo on nitisinone and stored at -80°C. Samples were diluted 1:3 with deionized water and stored at -80°C as three separate aliquots for analysis by methods 1-3. Metabolomic analysis was part of the diagnostic service to patients being seen at the NAC and with approval from the Royal Liverpool and Broadgreen University Hospital Trusts Audit Committee (audit no. ACO3836).

Mouse urine was from 18 (9 male, mean age 27±12 wk) *HGD*$^{-/-}$ BALB/c mice (16) bred from laboratory stocks at the University of Liverpool. Samples were obtained on a single-collection basis before treatment then after 1 wk on nitisinone, administered in all drinking water (0.4 mg/dL), supplied *ad libitum*. Mouse urine was collected onto cling film, pipetted into sample tubes and stored at -80°C. Analysis was performed following dilution of 1:9 with deionized water. Mouse breeding and dosing was authorized under the Animals (Scientific Procedures) Act, UK.

Samples were pooled for quality assurance in profiling experiments. For each sampling time point, a representative pool was created by pooling 20 µL of each individual urine sample. An additional overall pool was created separately for human and mouse experiments by pooling equal proportions of the pooled urine groups detailed above. Pooled human and mouse samples were stored and treated as above prior to analysis.

Analytical conditions

Chemicals

Water for mobiles phases was purified as above. Methanol, acetonitrile, isopropanol (Sigma-Aldrich), formic acid (Biosolve, Netherlands) and ammonium formate (Fisher Scientific, Germany) were LC/MS grade.

Equipment

Sample analysis was performed on an Agilent 1290 Infinity LC coupled to an Agilent 6550 QTOF-MS equipped with a dual AJS electrospray ionization source (Agilent, UK).

Chromatographic conditions

Three chromatographic methods were designed to separate different compound classes. Method 1: non-polar compounds. Method 2: a range of polar/non-polar compounds. Method 3: polar compounds. Metabolite standards were analyzed using all three LC-QTOF-MS techniques.

Method 1

A Zorbax Eclipse Plus C_{18} column (2.1x100 mm, 1.8 µm, Agilent) was maintained at 60°C (flow rate 0.4 mL/min). Mobile phases were (A) water and (B) methanol both containing 31.6 mg/dL ammonium formate and 0.1% formic acid. The elution gradient started at 5% B at 0-1 min increasing linearly to 100% by 12 min, held at 100% B until 14 min, returning to 95% A for 5 min.

Method 2

Method 2 employed the same conditions and elution gradient as method 1, but with an Atlantis dC_{18} column (3x100 mm, 3 µm, Waters, UK).

Method 3

A BEH amide column (3x150 mm, 1.7 µm, Waters) was maintained at 40°C (flow rate 0.6 mL/min). Mobile phases were (A) water and (B) acetonitrile (both containing 0.1% formic acid). The elution gradient started at 99% B decreasing linearly to 30% from 1-12 min, held at 30% B until 12.6 min, returning to 99% B for 3.4 min.

Sample injection volume was 1 µL for metabolite standards and human urine, and 2 µL for mouse urine. The autosampler was maintained at 4°C and the needle was washed with a solution of water:methanol:isopropanol (45:45:10 v/v) between injections.

Design of urine metabolic profiling experiments

Human and mouse urine was analyzed separately. Human samples were analyzed batch-wise using all 3 methods, negative followed by positive polarity. Mouse samples were analyzed by method 2 only due to limited sample volumes, in one batch comprising both polarities.

The analytical sequence of each profiling batch was designed according to published guidance (22). Each run commenced with 20 replicate injections of the overall pooled sample to condition the system. The order of individual samples was randomized computationally. Pooled samples were interspersed throughout the analytical sequence, every 10th injection. Injections of each sample group pool and the overall

pooled sample were also placed at the start (post-conditioning) and end of each analytical sequence.

Data acquisition and processing

Data acquisition and processing were performed using the MassHunter suite (Agilent). Data were acquired with Data Acquisition (build 06.00). Metabolite standards and urine samples were analyzed in both polarities, mass range 50-1700 (Supplemental File 3). Quality checks and processing of raw data were performed with Qualitative Analysis (build 07.00). Extracted ion chromatograms of reference masses were performed to check mass accuracy remained <5 ppm throughout the run. Binary pump pressure curves for injections across each analytical sequence were overlaid to check chromatographic reproducibility.

Compound signals were extracted from the standards data in Qualitative Analysis (build 07.00) by molecular formula using the 'find by formula' algorithm; mass window of theoretical accurate mass (calculated from molecular formula) ±5 ppm. Allowed ion species were: H^+ and Na^+ and additionally NH_4^+ for methods 1 and 2 (positive polarity); and H^- and additionally CHO_2^- for methods 1 and 2 (negative polarity). Charge state range was 1-2. Dimers were allowed. Separate AMRT databases were created for each method using PCDL Manager (build 08.00). Theoretical accurate mass, retention time (RT), molecular formula and METLIN chemical name were entered into each AMRT database for compounds that were retained and detected (databases publicly available via https://figshare.com/collections/_/4378235/0 (23)).

Urine profiling data were mined for signals matching AMRT database compounds using 'targeted feature extraction' (Profinder, build 08.00). Targeted feature extraction

uses the molecular formulae to extract and group spectral signals (*i.e.* adducts, multimers and isotopes), corresponding to individual database compounds. Feature extraction window; theoretical accurate mass ±10 ppm and database RT ±0.3 min. Allowed ion species were the same as specified above.

Detection of non-AMRT database compounds in urine

Urine data were also mined using Profinder for compounds not from generated AMRT databases but of interest for their predicted role in AKU/nitisinone metabolism. These compounds were associated with a) increased tyrosine: acetyl-L-tyrosine, γ-glutamyl-L-tyrosine and tyramine-sulfate; b) ochronotic pigment: 2,5-dihydroxybenzaldehyde, hipposudoric acid and norhipposudoric acid; or c) nitisinone metabolism: hydroxy-nitisinone, nitisinone and 2-nitro-4-trifluoromethylbenzoic acid. For non-AMRT compounds, the same RT (range <0.3 min) was required across samples. Compound identifications were based on theoretical accurate mass ±10 ppm.

Data quality control and statistical analyses

Several quality control (QC) filters were applied to AMRT-matched entities from each profiling batch. First, entities were retained if observed in at least two samples per experimental group (*i.e.* sampling time point). Data files were then exported from Profinder and imported into Mass Profiler Professional (build 14.5). 24 h creatinine excretions (Roche Diagnostics, Germany) were used as external scalar for individual human urine samples (average values across each sampling time point used for pooled samples). Creatinine values were not available for mouse samples; the signal

identified as creatinine by matched AMRT was used as external scalar for each sample using the peak area of the ^{13}C $[M+H]^+$ ion (^{13}C because ^{12}C creatinine signal was saturated) calculated in MassHunter Quantitative analysis (build 06.00).

Entities were then further filtered in Mass Profiler Professional based on data from pooled samples from each experiment. Entities were retained if observed in 100% of replicate injections for at least one sample group pool, and with peak area coefficient of variation (CV) <25% across replicate injections of all sample group pools.

Statistical analyses were performed in Mass Profiler Professional based on peak area. Human urine profiles were compared at 3, 12 and 24 mo on nitisinone with pre-nitisinone by one-way repeated-measures ANOVA. Mouse urine profiles were compared pre-nitisinone vs 1 wk on nitisinone by paired t-test. Benjamini-Hochberg false-discovery rate adjustment was used in all statistical significance testing. Fold changes (FC's) were calculated based on raw peak area. Principal component analyses employing four-component models were also performed on each filtered dataset.

Results

Analysis of metabolite standards

Signals representing the chemical formulae for standards in each injection were extracted from the data by theoretical accurate mass ±5 ppm. Only standards with RT >0.3 min after the column void volume were considered. 519/619 (83.8%) of the standards were retained sufficiently to be detected by at least one method. 116/619 (18.7%) and 226/619 (36.5%) of the compounds were retained sufficiently and

detected by one or two methods only, respectively, demonstrating the utility of combining data from the three chromatographic methods (Figure 2A). RT, accurate mass and charge state were entered into an AMRT metabolite database for each method for matching unknowns against.

Figure 2B-D shows the mass/RT distribution for compounds detected by each method. Figure 2B-D also highlights differences in selectivity for compounds from three example compound groups with different chemical properties: carbohydrates, amino acids and lipids (Supplemental File 4). Method 3 retained and separated highly polar compounds such as carbohydrates (20/26 detected, RT range 5-9.5 min). In methods 1 and 2, the same carbohydrates were weakly retained; for method 1 all carbohydrates detected eluted within the initial column void (24/26 detected, RT range 0.58-0.68 min) and for method 2 all carbohydrates detected eluted close to the initial void (23/26 detected, RT range 1.3-1.4 min). Method 1 showed some evidence of improved suitability for analysis of lipid-like compounds (11/11 detected, RT range 7.7-13.7 min) compared to methods 2 (8/11 detected, RT range 9.3-14.3 min) and 3 (7/11 detected, RT range 1.5-8.3 min), which detected fewer of these compounds within a narrower RT range overall. All three methods enabled detection of the amino acids, with method 3 appearing to show the most useful chromatographic resolution and retention (19/21 detected, RT range 5.4-8.1 min) compared to methods 1 (21/21 detected, RT range 0.6-2.8 min) and 2 (21/21 detected, RT range 1.0-4.6 min).

Identification of metabolites in pooled urine *by AMRT*

Unknown chemical entities were matched against the respective AMRT database generated for each method**:** accurate mass ±10 ppm, RT ±0.3 min. For data presented

here, analysis comprised two replicate injections of the overall pooled urine from patients with AKU from the start and end of each analytical run. Only unknowns obtained from both injections in positive or negative polarity and with single AMRT compound matches were retained in this analysis. Additional QC filters were then applied to ensure reproducibility across the run: CV <25% for peak area and RT between the two replicate injections. Table 1 summarizes matches retained for each method. No compounds were filtered out due to RT CV >25%. The maximum RT CV% change between the replicate injections across all analytical runs (each comprised 205 injections) was 4.1%.

Figure 3A shows that 203 unique compound matches were obtained from urine. Supplemental File 5 shows the matches obtained by methods 1-3. Fourteen AMRT matches were obtained by all three methods. An additional 61 AMRT matches were obtained by two methods, and 128 matches by only one of the methods, further supporting the increased coverage from the three methods in combination. Figure 3B-D shows the mass/RT distribution of the AMRT matches.

Table 1 and Supplemental Figure 2 show the number of AMRT matches obtained with narrower AMRT-matching windows. For each method, >50% of matches obtained with ±10 ppm and ±0.3 min post-QC remained with ±5 ppm and ±0.15 min.

Application of strategy to AKU: effect of nitisinone therapy on the urine metabolome

Figure 4A,B shows the overall study design and a representative example of AMRT compound signals extracted from the data respectively. Representative principal components analysis plots (Figure 4C) show clear separation between the AMRT-

matched profiles of urine pre- vs post-nitisinone for human and mouse, showing that our strategy captured key metabolite changes.

Thirty-five metabolites showed statistically significant changes ($p<0.05$, FC>2) in abundance after 3, 12 or 24 mo on nitisinone in humans or 1 wk on nitisinone in mice (Figure 5). An FC >2 was chosen in order to limit false discovery and focus on clear changes. In patients with AKU, 13 metabolites increased and 14 decreased. In *HGD*$^{-/-}$ mice, 12 metabolites increased and 6 decreased. Ten metabolites changed in the same direction in humans and mice (Figure 5). Together, the majority of these metabolite changes could be categorized into three main metabolic pathways: those of tyrosine, tryptophan and purine.

Interestingly, mouse data also showed clear separation by gender (principal component 1; x-axis Figure 4C). Histamine was the primary driver of this separation in positive polarity (principal component 1 loading; 0.42), which captured the effect of gender and explained 43% of the variation in the dataset. Histamine was significantly increased in female mice ($p<0.0001$, FC=16).

Alterations to non-AMRT database compounds post-nitisinone

Data showed alteration ($p<0.05$, FC>2) to metabolites with a predicted association to AKU/nitisinone metabolism that were not from AMRT databases (Supplemental Figure 3). 2,5-dihydroxybenzaldehyde, probably associated with ochronotic pigment, was decreased post-nitisinone. The tyrosine metabolites acetyl-L-tyrosine and γ-glutamyl-L-tyrosine and the nitisinone metabolite hydroxy-nitisinone were increased. These changes were observed in human and mouse urine.

Discussion

We have developed a strategy for comprehensive LC-QTOF-MS profiling with compound identification by three AMRT databases generated from metabolite standards. Application of this strategy enabled: a) identification of unknown chemical entities in complex biological matrix by AMRT; and b) identification of previously unreported changes to urinary metabolites and metabolic pathways following nitisinone treatment in AKU.

A limitation of LC-MS compound identification by public databases (24–28) is that data were acquired using different analytical techniques and parameters. The Metabolomics Standards Initiative has established levels of metabolite identification confidence (29,30). Only identification strategies, as reported here, that compared two or more orthogonal chemical properties (*e.g.* AMRT) with an authentic standard under identical analytical conditions achieved the highest identification confidence level. Identifications by public databases in which data were acquired under different analytical conditions can only achieve the second level of confidence ('putative identification'), even with two matched orthogonal chemical properties.

Combining data from three LC techniques enhanced the number of unique urine AMRT matches obtained, improving coverage of the metabolome. Methods 1 and 2 showed some similarity in analyte retention (Figure 2B-D). Method 2 was used because the Atlantis dC_{18} column was previously shown to chromatographically resolve metabolites of the tyrosine pathway in patients with AKU (31-32). It also provided greater overall retention of polar metabolites compared to a standard C_{18} column due to endcapping of bi-functionally bonded C_{18} stationary phase. Method 1

was included here to improve peak capacity owing to the increased theoretical plates provided by the smaller 2.1 µm column particle size. Moreover, methods 1 and 2 yielded 39 and 57 AMRT matches respectively that were exclusive to each method (Figure 3A).

The congenital defect that causes AKU directly affects tyrosine catabolism. However, AKU is multi-systemic (9) and the wider metabolic consequences of the disease and nitisinone treatment have not been systematically studied. The strategy was applied to AKU, but given the range of primary and intermediary metabolism covered by the AMRT compounds, it could be applied to study metabolism in any disease. The data show that nitisinone alters tyrosine and tryptophan metabolism, and support alteration to the purine metabolic pathway. The changes observed comprise increased and decreased concentrations within the same pathways, suggesting that nitisinone has complex, wide-ranging effects on metabolism. Nitisinone is licensed to treat patients with hereditary tyrosinaemia type-1 (HT-1, OMIM 276700), another congenital disease of tyrosine metabolism, and is a promising HGA-reducing agent in AKU. In nitisinone-treated HT-1 and AKU it is established that circulating tyrosine increases markedly (11,13–15,17–21). Tyrosine is the precursor for catecholamines, thyroid hormones and melanin (Figure 1), suggesting that increased tyrosine substrate has potential to dysregulate these pathways. In HT-1 there are concerns that hypertyrosinaemia may contribute to neurodevelopmental delay (19,33-36).

Application of our profiling strategy identified a number of previously unreported metabolite changes post-nitisinone. Increased 3-(4-hydroxyphenyl)lactic acid is probably directly related to elevated tyrosine and/or its clearance in urine. Changes a) homovanillic acid and the trace amine tyramine and b) L-tryptophan represent further

357 alterations to dopamine and tryptophan-serotonin metabolism (respectively) than
358 those previously reported. L-tryptophan is the precursor for serotonin, and a post-
359 nitisinone increase in the serotonin metabolite 5-hydroxyindoleacetic acid was
360 previously reported in HT-1 cerebrospinal fluid (37) and AKU urine (20), although not
361 observed here. Xanthurenic acid and L-kynurenine were increased and decreased
362 respectively, indicating for the first time that the kynurenine pathway, originating from
363 tryptophan, is altered by nitisinone. Also, indoxyl sulfate, a metabolite of the
364 indolepyruvate pathway from tryptophan, was decreased. Alterations to
365 indolepyruvate metabolism are previously reported in nitisinone-treated AKU plasma;
366 carboxaldehyde, indole-lactate and indole-pyruvate increased, and *in vitro* studies
367 indicated this as a direct consequence of increased 3-(4-hydroxyphenyl)pyruvic acid
368 (38). This is the first report of alterations to purine metabolism following treatment with
369 nitisinone. Decreased 3,5-cyclic-AMP and xanthosine were unexpected but replicated
370 in mice.

371 The remaining metabolite changes have been previously reported following nitisinone
372 treatment; decreased HGA and increased tyrosine, acetyl-L-tyrosine, γ-glutamyl-
373 tyrosine, 3-(4-hydroxyphenyl)pyruvic acid and 3-methoxytyramine. Decreased HGA
374 and increased tyrosine are well-known consequences of nitisinone, and observation
375 of these changes in each analytical run (tyrosine in positive and HGA in negative
376 polarity) supports the analysis, data extraction workflow, and validity of the data.
377 Increased acetyl-L-tyrosine and γ-glutamyl-tyrosine have been previously reported in
378 nitisinone-treated AKU and were proportional to tyrosine elevation (39). Increased 3-
379 methoxytyramine was previously reported in AKU urine (20); it is a metabolite of
380 dopamine metabolism and derived from tyrosine via decarboxylation of
381 dihydroxyphenylalanine to dopamine.

Ten AMRT-matched metabolite changes were observed in human and mouse: 4-hydroxyphenylacetic acid, 3,(4-hydroxyphenyl)lactic acid, 3-(4-hydroxyphenyl)pyruvic acid, L-tyrosine, phenylacetic acid and tyramine increased post-nitisinone; 2-hydroxy-4-(methylthio)butyric acid, 3,5-cyclic AMP, HGA and xanthosine decreased post-nitisinone. The concordance between human and mouse data supports the approach and validity of the data. For mice, it was possible to control potentially confounding factors that could affect metabolism, such as diet and genetic diversity. This increases likelihood that observed changes are attributable to nitisinone. Reduced phenotypic variation could explain the prominent gender difference observed for mouse only; histamine was particularly elevated in urine from females, as previously reported in the literature for rats (40,41) but not mice to our knowledge.

The limitations of this study are as follows. First is the relatively small sample size for a clinical metabolomics study (25 patients). However, AKU is a rare disease and the repeated-measures design enhanced statistical power. Second, data represent changes observed in urine not serum, which more closely reflects internal homeostasis. The urine metabolome is a composite of products from endogenous metabolism, diet, drugs and the gut microbiome. Urine does however give a valuable indication of the metabolic fate of the increased circulating tyrosine post-nitisinone. Further metabolomic analyses on fluids and tissues from other biological locations are required to achieve more detailed, compartment-specific data, for example cerebrospinal fluid to directly study the impact of nitisinone-induced tyrosinaemia on the central nervous system. Third, three chromatographic methods were used, however this may not always be feasible. For large-scale studies, it may be pragmatic to use two of the methods to reduce analytical and processing time.

406 In conclusion, we have developed a targeted LC-QTOF-MS strategy for
407 comprehensive coverage of the metabolome with compound structure identification
408 using three AMRT databases, which are publicly available. Application of the approach
409 to AKU has advanced our knowledge of the wider metabolic consequences of
410 nitisinone, demonstrating the potential of our method as a metabolic phenotyping
411 strategy more generally.

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Table 1. Number of **accurate mass retention time** (AMRT) matches obtained by the three methods and retained following quality control filtering and decreasing AMRT window size during feature extraction.

Feature extraction window	Filtering step	Number of urine AMRT matches					
		Method 1		Method 2		Method 3	
		Positive	Negative	Positive	Negative	Positive	Negative
Accurate mass 10 ppm, RT 0.3 min	None	274	153	248	105	111	74
	(1) Unique AMRT's only, manual curation	80	38	121	43	70	37
	(2) Abundance QC: peak area CV <25% between replicates	75	36	107	35	59	30
	(3) RT shift QC: RT CV <25% between replicates	75	36	107	35	59	30
Accurate mass 5 ppm, RT 0.15 min	Steps (1) - (3)	44	31	65	25	38	22
Accurate mass 2.5 ppm, RT 0.075 min	Steps (1) - (3)	17	21	22	12	14	13

RT, retention time; Positive, positive ionization mode; Negative, negative ionization mode.

Figure Legends

Figure 1. Tyrosine catabolic pathway with reference to its defect in alkaptonuria and treatment with nitisinone. Lack of the enzyme homogentisate 1,2-dioxygenase increases concentrations of circulating homogentisic acid by preventing its conversion to maleylacetoacetic acid. It is previously established that nitisinone has the upstream consequence of increased concentrations of tyrosine, 3-(4-hydroxyphenyl)pyruvic acid and 3-(4-hydroxyphenyl)lactic acid as a result of the metabolic block induced by nitisinone.

Figure 2. Summary of the data acquired from analysis of metabolite standards. A, Venn diagram summarizing the number of compounds retained and detected by methods 1-3, both alone (non-overlapping sections) and in combination (overlapping sections). B-D, Mass/RT scatter plots for metabolite standards detected with the three analytical methods, showing the different selectivities of the methods for compounds from different chemical classes.

Figure 3. Summary of compounds identified from analysis of urine by accurate mass retention time (ARMT). A, Venn diagram summarizing the number of AMRT matches obtained for methods 1-3. B-D, Mass/RT scatter plots for AMRT compound matches obtained from analysis of urine by the three analytical methods.

Figure 4. Application of the profiling strategy to alkaptonuria (AKU). A, Urine was obtained from patients with AKU and *HGD*$^{-/-}$ mice pre- then post-nitisinone therapy. B, Representative urine profiles (top) and extracted signals (bottom) for compounds identified by accurate mass retention time. C, Principle component analysis showing alteration to human (left) and mouse (right) urine metabolomes post-nitisinone. x, y and z axes represent components 1, 2 and 3 respectively.

Figure 5. Urinary metabolite changes identified post-nitisinone in alkaptonuria by application of the profiling strategy. Red and blue indicate increases and decreases respectively. Fold changes (FC's) are indicated in brackets and were calculated from raw peak area.